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Topical Application of Autologous Leukocyte-Rich Platelet-Rich Plasma with a High Concentration of Monocytes in Patients with Recalcitrant Ischemic Diabetic Foot Ulcers: Efficacy, Safety and Economic Evaluation.

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Topical Application of Autologous Leukocyte-Rich Platelet-Rich Plasma with a High Concentration of Monocytes in Patients with Recalcitrant Ischemic Diabetic Foot Ulcers: Efficacy, Safety and Economic Evaluation

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ABSTRACT: BACKGROUND: Patients with diabetic foot ulcers (DFU) and chronic limb-threatening ischemia (CLTI are at higher risk for major amputations and mortality. Aim of this study was to assess the safety and cost-efficacy of intramuscular injection of autologous monocyte-rich PRP platelet-rich plasma Tropocells® PRP (Estar Medical, Holon, Israel) in diabetic patients with chronic limb-threatening ischemia (CLTI) in which revascularization was not feasible. METHODS: a retrospective study was carried out on a series of patients with type 2 diabetes, grade Texas 3C or D DFU, and CLTI. All patients had undergone at least a previous revascularization and were allocated in a surgery waiting-list for major amputation. According to the data provided by the manufacturer (https://www.zuccatobiotech.it/index.php/prodotti/tropocells), the monocyte-rich PRP kit obtained a platelet-rich plasma with a high concentration of peripheral blood mononuclear cells (with a proportionately lower amount of lymphocytes), a low concentration of neutrophils and an almost complete depletion of red blood cells. All patients underwent a procedure of local infiltration of autologous mononuclear cells through multiple perilesional and intramuscular injections of 10 mL PBMNC suspensions (0.2–0.3 mL in boluses) performed below the knee along the relevant vascular axis (anterior tibial artery and posterior tibial artery) at intervals of 1–2 cm and to a mean depth of 1.5–2 cm, using a 21 G needle. The procedures were performed according to the instructions of the manufacturer and were repeated at least two times (maximum 3 times) at intervals of 30 days. The principal endpoint was a composite of $TcPO_2$ values at the first toe ≥30 mmHg and/or $TcPO_2$ increase of at least 50% from baseline and/or ulcer healing. Secondary endpoints were individual components of the primary endpoint, quality of life, any adverse events, direct costs. All the endpoints were evaluated at 26 weeks. RESULTS: Out of 21 enrolled patients, 13 (61.9%) reached the primary endpoint, whereas three patients underwent a major amputation, and two patients died. The median TpCO2 was significantly increased, and pain was significantly reduced. The overall mean and median cost per patient were 9,255±7,328€ and 4,001 [2,991;7,565]€, respectively. CONCLUSIONS: the use of PBMNCs implants in patients with DFU and no-option CLTI seems to be efficacious in reducing the risk for major amputation and improve ulcer healing.

Keywords: diabetes mellitus; foot ulcer; cell-therapy; chronic limb-threatening ischemia; economic evaluation

Introduction

DFU (Diabetic Foot Ulcers) represent one of the main causes of lower limb adverse events in patients with diabetes[1], such as major amputation[2] and mortality[3], particularly in case of concomitant peripheral artery disease (PAD)[1]. Peripheral artery disease (PAD) and diabetic neuropathy, which are frequently concomitant[4] are well-known risk factors for the development of DFU. Chronic limb-threatening ischemia (CLTI)[5] represents the most advanced form of PAD, and it is associated with lower ulcers healing[3], and a higher risk for unfavourable outcomes[2,3].

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The gold standard for the treatment of ischemic DFU is artery revascularization, either through endovascular angioplasty or bypass surgery[6]; however, in a significant proportion of patients, such procedures are not feasible, or not effective in preventing amputation[7]. For these patients, autologous cell transplant therapy has emerged as a promising approach[7]: it consists on the administration of mesenchymal stem cells, blood marrow mononuclear cells, or peripheral blood mononuclear cells (PBMNCs), usually through intramuscular injection, intravenous infusion, direct injection into target tissue or muscle, or delivery through a biomaterial or scaffold[7], depending on the cell type, stage of the disease, and treatment goal. Autologous transplant cell therapy has shown favourable effects on pain, transcutaneous oxygen tension, ulcer healing, major amputation, and mortality[8], by stimulating neo-angiogenesis, vasculogenesis and tissue repair[7]. Proposed mechanisms are the paracrine activities of growth factors, cytokines, and messenger molecules[9,10]. Bone-marrow derived mononuclear cells, however, are obtained through invasive, painful and time-consuming extraction procedures, which require hospitalisation. On the other hand, peripheral blood mononuclear cells (PBMNCs), which do not present such disadvantages, have shown similar efficacy on angiogienesis[8]; in addition, epidermal regenerative effects with PBMNCs have been detected, through the activation of macrophage colony-stimulating factors[11], possibly increasing the ulcer healing rate in patients affected by DFU[11-13]. Some randomized and non-randomized studies in patients with DFU have shown the efficacy of PBMNCs obtained through prior administration of granulocyte colony-stimulating factor (G-CSF), to mobilize hematopoietic progenitor cells from bone marrow[7,14-17]; this procedure, although less invasive, may harbour several adverse events, such as pyrexia, bone pain, back pain, neutropenia and febrile neutropenia[14], and more rarely risks of embolism[14].

Different techniques to obtain PBMNCs have also been tested in a few studies in patients with DFU, such as leukocyte and platelet-rich fibrin patch[18], purified circulating fibrocytes[19], or monocytes obtained by filtration[13,20-23].

In this paper, we explore the effects of mononuclear cells obtained with a new technique described for the first time by Mercuri et al. for the treatment of vitiligo[24]. It is a leukocyte-rich platelet-rich plasma with a high concentration of monocytes (hereinafter defined as monocyte-rich PRP) obtained by blood sample centrifugation. To our knowledge, no studies have explored the effects of this new formulation of autologous mononuclear cells in patients with DFU. We therefore decided to test monocyte-rich PRP for the treatment of DFU in an uncontrolled nonrandomized retrospective study.

Patients and Methods

The present retrospective analysis was performed on a consecutive series of patients with ischemic Texas 3 DFU, with a duration of at least 6 months, undergoing the implantation of PBMNCs from peripheral blood at the Diabetic Foot Unit of Careggi Hospital, Florence, Italy, between January 1st, 2022 and September 1st, 2022. All patients were candidates for elective minor or major amputations and allocated in a surgery waiting-list.

The study protocol was approved by the local ethical committee (Protocol number SPE_22580), and informed consent was obtained from all patients before the inclusion in the analysis.

Patients were included if they fulfilled the following criteria:

1) Diagnosis of diabetes mellitus
2) Age > 18 years;
3) DFUs grade Texas[25] 3C or D with a duration >26 weeks;
4) Peripheral artery disease was diagnosed in case of a stenosis of at least 50% in one of the lower limb arteries and TcPO2 values at foot level < 60 mmHg[27]
5) At least one previous attempt to revascularization and no longer eligibility for further revascularizations due to technical difficulties to overcome vessel obstruction and/or high number of comorbid conditions.
6) Absence of severe infection according to the PEDIS classification system (PEDIS<3[26])
7) Absence of severe anaemia (Hb > 8 g/dL)
8) Absence of coagulation disorder/thrombocytopenia (PLT > 50,000/L)

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9) Absence of active cancer/leukaemia or lymphoma or haematological disease
10) Being able to sign informed consent.

All patients received a multidisciplinary evaluation (including diabetologists, vascular surgeons and interventional cardiologists) to explore the possibility of a new lower limb revascularization, and to discuss the indication to perform implantation of perilesional and perivascular monocytes.

Baseline data collection

Demographic and clinical data were collected from clinical records, including a medical history with detailed information on concomitant complications, medical conditions, current pharmacological treatment, and duration of diabetes and foot ulcers. At the first visit, following a standard procedure of our Clinic, all patients underwent a complete physical examination, and blood sample was collected for HbA1c, glycaemia, creatinine, total cholesterol, HDL-cholesterol, and triglycerides. Ulcers area was measured using MolecuLight i:X ®. In the case of two or more lesions, the largest ulcer was taken into account. Diagnosis of diabetic neuropathy was performed measuring vibratory perception threshold with a biothesiometer (METEDA, San Benedetto del Tronto, Italy) and monofilament testing 10g. Ulcers were classified according to the University of Texas score[25].

Pain and quality of life were assessed using a visual analogue scale (VAS) ranging from 0 to 10 and from 0 to 100 for pain and quality of life, respectively.

Transcutaneous pressure of oxygen (TcpO2; Radiometer Medical ApS; Brønshøj, Denmark) at the hallux and ankle level and ankle-brachial index (ABI; or toe brachial index) was measured and an echo-color doppler examination of lower-limb arteries was performed.

Renal failure was defined as a reported previous diagnosis of renal failure, or as serum creatinine >1.5 mg/dl. Ischemic Heart Disease (IHD) and cerebrovascular disease were diagnosed when patients reported previous myocardial infarction/angina or stroke/transient ischemic attack. Comorbidity was assessed through the calculation of Charlson's comorbidity score (CCS).

Ulcer treatment and PBMNC collection

All patients received the same standard therapy according to IWGDF guidelines[28]: surgical debridement, local dressings, foot offloading, antiplatelet drugs, antibiotic therapy in case of infection, and pain relief therapy.

The preparation system used to obtain the monocyte-rich PRP was the Tropocells® PRP (Estar Medical, Holon, Israel). According to the data provided by the manufacturer (https://www.zuccatobiotech.it/index.php/prodotti/tropocells), the monocyte-rich PRP kit obtained a platelet-rich plasma with a high concentration of peripheral blood mononuclear cells (with a proportionately lower amount of lymphocytes), a low concentration of neutrophils and an almost complete depletion of red blood cells. All patients underwent a procedure of local infiltration of autologous mononuclear cells through multiple perilesional and intramuscular injections of 10 mL PBMNC suspensions (0.2–0.3 mL in boluses) performed below the knee along the relevant vascular axis (anterior tibial artery and posterior tibial artery) at intervals of 1–2 cm and to a mean depth of 1.5–2 cm, using a 21 G needle. The procedures were performed according to the instructions of the manufacturer and were repeated at least two times (maximum 3 times) at intervals of 30 days. All procedures were performed in an operating room with anesthesiologic support (midazolam iv and/or peripheral nerve block).

Follow-up data

Patients were evaluated at baseline, one, three, and six months after the first implantation and the following parameters were recorded:

1) Healing rate
2) Pain (using VAS scale from 0 to 10)
3) TcPO2 at the 1st toe
4) Vital status

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5) Major and minor amputation rate
After 6 months:
1) Quality of life

Endpoints

The primary endpoint of the study was a composite of the following items at 26 weeks:

1) healing of the ulcer
and/or
2) $TcPO_2$ at the first toe ≥30 mmHg (only for patients with baseline values< 30 mmHg) and/or increase of at least 50% of TcPO2 in comparison with baseline values

Secondary outcomes evaluated at each time-point were:

- individual components of the primary endpoint;
- 6-month quality of life;
- any serious and non-serious adverse events;
- direct costs at one year.

Complete healing was defined as full epithelialization of the wound (including when obtained after minor amputation), confirmed by a subsequent visit after 7 days. Minor amputations were performed, as recommended by international guidelines[28] only with distal TcPO2 >= 30 mmHg or in case of a 50% increase of TcPO2 compared with basal values; minor amputation was considered as limb rescue, and it was defined as any amputation performed below the ankle. Major amputation was defined as a surgical procedure performed above the ankle.

Economic assessment

The economic assessment was performed considering the perspective of the local health system, thus considering only direct healthcare costs and including costs associated with healthcare resources used all over the follow-up and extracted from clinical records. In detail, direct costs included specialist visits, diagnostic procedures, hospital admissions (related to diabetic foot), major and minor amputations, antibiotic therapy, grafts, and off-loading orthesis. Costs for hospitalizations were estimated on the basis of established regional tariffs (https://www.salute.gov.it/portale/temi/p2_6.jspid=3662&area=programmazioneSanitariaLea&menu=vuoto), i.e. tariffs established for the diagnosis related group (DRG) associated with each episode for hospital admissions (either day-hospital or full-length stay) and recorded in clinical records; similarly for costs related to specialistic visits and outpatient procedures performed (e.g. RX, MRI, laboratory exams, ecc.). The cost of antibiotic therapy was estimated considering ex-factory prices(https://www.salute.gov.it/portale/temi/p2_6.jsp?id=3662&area=programmazioneSanitariaLea&menu=vuoto),while current market prices were used to value costs for orthopaedic shoes/orthesis. The health economic analysis performed tried to estimate costs born to the healthcare system, mainly using tariffs related to different healthcare services, over one year. As discounting typically require collection of data over different time point to give different value to both costs and health outcomes that are predicted to occur in the future because they are usually valued less than present costs, given the time frame considered in our analysis we decided to not apply any discount rate. All costs were referred to 2020 and are reported in Table 1S and 2S of Supplementary Materials.

Statistical analyses

Statistical analysis was performed on SPSS 25.0. Data were expressed as mean ± standard deviation (Std.dev), or as median (25th-75th percentile), depending on their distribution. Comparisons between groups were performed using Student's t-test for independent samples or Mann–Whitney U test as appropriate. Chi-square and Fisher exact tests were used for between-group comparisons of categorical variables as appropriate.

Results

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The principal characteristics of patients are summarised in Table 1. Briefly, the whole cohort was composed of 21 patients (7 women, 33.3%), aged 71.8 years on average, and affected by ischemic DFU (47.6% Texas 3C, and 52.4% Texas 3D grade). Most DFU involved the forefoot (85.7%). Gangrene was present in 24% of cases. Median $TcPO_2$ at the hallux level at baseline was 23.7 mmHg and duration of ulcers was on average 345 days.

Following our internal protocol all patients, except four, underwent two infiltrations of PBMNCs; one patient received three infiltrations due to an incomplete response to the treatment, and the other two patients required a major amputation before undergoing the second infiltration.

The primary 26-week composite endpoint was achieved in 13 patients (61.9 %). Nine patients (42,9%) healed within 26 weeks and 4 non-healed patients showed a distal $TcPo_2$> 30 mmHg. Out of 9 healed patients, 3 (33,3%) required a minor amputation. Two patients underwent major amputations and 3 patients (one after healing) died.

A progressive increase of median $TcPO_2$ in comparison to baseline values was observed, which reached significance at 3 and 6 months, when values of 33.7 and 37.2 mmHg, respectively, were reached (Table 2).

A statistically significant reduction of pain measured through the VAS scale was observed at any time-point (Table 2).

Quality of life measured at six months showed a nonsignificant trend toward increase of 10/100 points from baseline (p=0.09, table 2).

No adverse events related to the treatment were observed during follow-up and only three patients reported pain immediately after the procedure (median entity 3.8 VAS scale points) which completely disappeared in a few minutes without requiring any specific treatment.

A formal analysis of direct costs sustained during the 6-month follow- is reported in **Table 3.** The overall mean and median cost per patient were 9,255±7,328€ and 4,001[2,991;7,565]€, respectively.

Table 1. Main anthropometric and demographic characteristics of the enrolled cohort and of observed ulcers.

	Cases **(n= 21)**
Age(years)	71.8±12.7
Gender (women, %)	7 (33.3%)
Body Mass Index(kg/m^2)	24.8±3.9
Diabetes type 2 (%)	20 (95.2)
Diabetes duration(years)	23.4 ±10.1
Medical history and risk factors (n, %)	
Charlson's score index	5.0[3.0-6.5]
Peripheral artery disease	21 (100.0%)
Neuropathy	21 (100.0%)
Retinopathy	11 (52.4%)
Chronic renal insufficiency	12 (57.1%)
Dialysis	3 (1.3%)
Ischemic heart disease	10 (47.6%)
Hearth failure	4 (26.7%)
Heart failue	7 (33,3)
Ictus	3 (8.6%)
Charcot disease	4 (19.0%)
Connective tissue diseases	2 (9.5%)
Malignancies (< 5 years)	0 (0.0 %)
Cognitive impairment	2 (9.5%)
Smokers	1 (6.6%)
Dyslipidaemia	20 (95.2%)

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Hypertension	20 (95.2%)
Number of previous revascularization (%)	
1	14 (76.7)
2	4 (19.0)
3	1 (4.8)
4	1 (4.8)
5+	1 (4.8)
Laboratory parameters	
HbA1c (mmol/mol)	53.6± 12.2
Creatinine(mg/dl)	1.2 [0.85; 1.74]
LDL-Cholesterol(mg/dl)	58.7±34.9
Pharmacological treatment (n, %)	
Insulin	11 (52.4%)
Glucose-lowering agents	14 (66.7%)
Antiaggregants	16 (76.2%)
Anticoagulants	13 (61.9%)
Statins	20 (95.2%)
Main ulcers' characteristics	
Duration (days)	345 [200; 444]
Site	
Forefoot	18 (85.7)
Midfoot	1 (4.8)
Hindfoot	2 (9.5)
Area (cm^2)	1.2 [0.3; 3.4]
TEXAS (%)	
3C	10 (47.6%)
3D	11 (52.4%)
Gangrene (%)	5 (23.8)
Osteomyelitis (%)	12 (57.1)
TcPO2 (mmHg)	
Hallux	23.7 [4.3;36.3]
Ankle	37.3 [24;47.7]
Pain (VAS 0-10)	5.0 [1.0; 6.0]
Quality of life (VAS 0-100)	50 [31; 65]

Table 2. Median values of distal $TcPO_2$ (basis hallux), perceived pain, and quality of life at 0, 1, 3, and 6 months.

Month	0	1	3	6
N Patients	21	21	19	16
$TcPO_2$ (mmHg)	23.7[4.3;36.3]	29.7[3.2;43.1]	33.7[7.1;45.0]*	37.2[10.1;49.9]*
Pain (0-10 pts)	5[1;6]	4[0;5]*	0[0;4.5]*	0[0;2]*
Quality of life (0-100 pts)	50[27;60]	NE	NE	60[30;70]

* $p< 0.05$ from baseline; NE: Not Evaluated; Pain was measured through the VAS scal; Quality of Life was measured using.

Table 3. Average costs during the follow-up of 6 months.

	Mean Std. Dev.	Median [interquartile]
Minor amputations/grafts	395±502	0 [0;800]
HA for FRP	1,508±1,908	0 [0;5,003]
Outpatient visits and laboratory exams	590±311	574 [284;721]

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Major amputations	3,310±7,002	0[0;0]
Antibiotics	572±776	0[0;51]
PBMNCs	2,880±1,018	2,001[1,408;3,021]
Total costs	**9,255±7,328**	**4,001[2,991;7,565]**

HA: hospital admission; FRP: foot-related problems; Std: Standard; dev: deviations.

Discussion

Recalcitrant DFU is a challenging condition often leading to major adverse limb events, such as major amputation[2] and mortality[3].

Multiple factors predicted longer time-to-ulcer-free in patients with DFUs, such as older age, neuropathy, PAD, ulcer size, depth of ulcer, and infection[29]. In particular, PAD seems to have a wide negative impact on prognosis particularly in the first 6 months after first referral to Diabetic Foot Units, suggesting that timely interventions could reduce the risk of major adverse limb events[29]. In our cohort, all patients received the best medical treatment for PAD according to international guidelines[27] and at least one attempt of revascularization was performed; in addition, all patients were judged no longer eligible for revascularization due to technical difficulties in overcoming vessel obstruction and/or a high number of comorbid conditions. We therefore decided to use PBMNC as an attempt of increasing ulcer healing rate, with a 42.9 % of patient reaching ulcer healing.

The interest for cell-based therapies in ischemic diabetic foot ulcers is growing [8,12], but several issues still need to be fully addressed to optimise their safety and efficacy: among those, the selection of the most appropriate cell type, dose, and delivery method, as well as the optimization of the therapeutic window, timing, and endpoints[30]. We decided to use a technique which allows the obtainment of peripheral mononuclear cells without the use of G-CSF, in order to minimize the delay in treatment deliver, the risk for adverse events, and to reduce direct costs, due to avoiding hospitalization, G-CSF treatment , and mobilization procedures.

We are therefore confident that our study, although limited by its retrospective and uncontrolled nature and the small sample size, can provide some insights on this topic and be of help for clinicians involved in the treatment of recalcitrant ischemic DFU. In fact, the obtained results (i.e., the number of healed patients, the increase of $TcPO_2$ values, the reduction of pain, and the relatively low rate of major amputations) are encouraging and of help as a hypothesis-generating research. In the present study, we have also assessed direct costs sustained for the treatment of these patients, which are relevant, but significantly lower than that needed for major amputations.

In conclusion, despite these promising results, further studies (in particular, randomised controlled trials) are needed to elucidate the mechanisms, optimise the procedures, assess the cost-effectiveness and validate the safety and efficacy of cell-based therapies for recalcitrant DFU.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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